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Chromatin structure is required to block transcription of the methylated herpes simplex virus thymidine kinase gene

(DNA methylation/chromatin reconstitution *in vitro*/microinjection/thymidine kinase gene expression)

G. BUSCHHAUSEN, B. WITTIG, M. GRAESSMANN, AND A. GRAESSMANN

Institut fuer Molekularbiologie und Biochemie, Freie Universitaet Berlin, Arnimallee 22, 1000 Berlin 33, Federal Republic of Germany

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ABSTRACT **Inhibition of herpes simplex virus (HSV) thymidine kinase (TK) gene transcription (pHSV-106, pML-BPV-TK4) by DNA methylation is an indirect effect, which occurs with a latency period of ≈8 hr after microinjection of the DNA into TK⁻ rat 2 and mouse LTK⁻ cells. We have strong evidence that chromatin formation is critical for the transition of the injected DNA from methylation insensitivity to methylation sensitivity. Chromatin was reconstituted *in vitro* by using methylated and mock-methylated HSV TK DNA and purified chicken histone octamers. After microinjection, the methylated chromatin was always biologically inactive, as tested by autoradiography of the cells after incubation with [^{3}H]thymidine and by RNA dot blot analysis. However, in transformed cell lines, reactivation of the methylated chromatin occurred after treatment with 5-azacytidine. Furthermore, integration of the TK chromatin into the host genome is not required to block expression of the methylated TK gene. Mouse cells that contained the pML-BPV-TK4 chromatin permanently in an episomal state also did not support TK gene expression as long as the TK DNA remained methylated.**

The question of how gene expression is regulated in mammalian cells is still a major unsolved problem of molecular and cellular biology. A large body of evidence indicates that regulation of DNA transcription is not directed by a uniform principle, but that multiple strategies are used by the cells. These strategies involve the interplay of DNA with a variety of macromolecules, the change of DNA and chromatin structure, and DNA modification (1–7). A well-analyzed modification step is DNA methylation. In mammalian cells, methylation occurs almost exclusively at the cytosine in the CpG dinucleotide sequence. There are several lines of evidence that support the assumption that the state of DNA methylation is critical for gene expression. It has been shown that different genes are more extensively methylated in their inactive state than in their active state. A more direct link between DNA methylation and gene expression was obtained by DNA transfection and microinjection experiments. Some genes methylated *in vitro* were found to be inactive after their transfer into recipient cells (for review, see refs. 8 and 9). We have documented the existence of a second class of genes that are methylation insensitive. Following microinjection of fully methylated simian virus 40 (SV40) or polyoma virus DNA into culture cells, early viral gene expression and DNA replication occurred with high efficiency (10, 11). Tanaka *et al.* (12) described a third category of genes in which hypermethylation precedes their expression.

To obtain a more detailed understanding of the role of DNA methylation in gene expression, we analyzed the biological activity of the methylated herpes simplex virus (HSV) thymidine kinase (TK) gene. In previous experiments, we have demonstrated that DNA methylation *per se* is insufficient to block HSV TK gene expression early after injection (13). In this investigation, we present evidence that shows inhibition of transcription by DNA methylation requires chromatin formation in the recipient cells. *In vitro* reconstituted methylated HSV TK chromatin was always inactive after microinjection into TK⁻ rat and mouse cells. In contrast, mock-methylated HSV TK minichromosomes were found to be fully active as tested by [^{3}H]thymidine incorporation and by RNA dot analysis. Reactivation of the inactive methylated chromatin was possible following exposure of the cells to 5-azacytidine.

MATERIALS AND METHODS

HSV TK Plasmids Used. (*i*) pHSV-106 (14) contains the TK gene in the *Bam*HI site of pBR322. (*ii*) pML-BPV-TK4 (15) contains the TK gene in the *Bam*HI site and a 5.5-kilobase fragment of the bovine papilloma virus (BPV) DNA cloned at the *Hin*dIII and *Bam*HI sites of pML.

Preparation of Histone Octamers and Chromatin Reconstitution. Chicken nuclei were isolated as described (16, 17). Nuclei (3×10^{10}) suspended in 0.25 M sucrose solution were diluted with an equal volume of 0.25 M HCl/0.2 M NaCl solution. After 30 min on ice, the suspension was centrifuged at 10,000 × *g* for 20 min and the supernatant was dialyzed twice against water and then freeze-dried. Core histones were isolated by treatment of the freeze-dried material with 5% perchloric acid (0.1 ml/mg) and centrifugation at 10,000 × *g* for 15 min. The pellet was reextracted in 2 ml of 0.25 M HCl and dialyzed twice against 0.25 M HCl and three times against water. The freeze-dried core histones (free of histones H1 and H5) can be stored at −20°C.

To reconstitute histone octamer (17), the freeze-dried core histones were suspended in 8 M urea/1% (vol/vol) 2-mercaptoethanol to a final concentration of 4 mg/ml. After incubation for 2 hr at room temperature, the suspension was dialyzed against 2 M NaCl/1% 2-mercaptoethanol/10 mM Tris·HCl, pH 7.4. The probe was then applied to a column (Sephadex 6B; 1 m × 2.5 cm), and eluted with 2 M NaCl/0.1 mM phenylmethylsulfonyl fluoride/10 mM Tris·HCl, pH 7.4, and the fractions containing the octamers were collected by measuring the absorbance at 280 nm. Final concentration was ≈0.1 mg/ml.

For chromatin reconstitution, 10 μg of DNA was mixed with 10 μg of reconstituted octamers in a final volume of 100 μl and dialyzed twice against 100 mM KCl/20 mM NaCl/0.25 mM EDTA/10 mM 2-mercaptoethanol/0.1 mM phenylmethylsulfonyl fluoride/10 mM Tris·HCl, pH 7.4, for 48 hr.

For methylation, 5 μg of the HSV TK plasmid DNA (pHSV-106, pML-BPV-TK4) was incubated with *Hpa* II methylase under conditions recommended by the manufacturer (Boehringer Mannheim). Completion of methylation

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Abbreviations: HSV, herpes simplex virus; TK, thymidine kinase; BPV, bovine papilloma virus; SV40, simian virus 40.

was checked by incubation of the DNA with the *Hpa* II and *Msp* I endonucleases and blot analysis as described (13).

Cells, Microinjection, and Autoradiography. For all experiments, TK$^-$ rat 2 and mouse LTK$^-$ cells were used. Details of the microinjection technique are described elsewhere (18). To assay the HSV TK enzyme activity at the cellular level, [^{3}H]thymidine (Amersham; specific activity, 40–60 Ci/mmol; 1 Ci = 37 GBq) was added to Dulbecco's modified Eagle's medium plus 5% fetal calf serum to a final concentration of 1 μCi/ml for 24 hr. After incubation, cells were washed with phosphate-buffered saline (PBS) and fixed in a mixture of acetone/methanol (2:1) for 10 min, covered with Kodak nuclear track emulsion NTB, exposed for 1 week at 4°C, developed (Kodak D19), and fixed with Kodak Unifix. G-418 selection (0.5–1 mg/ml) was started 1 day after microinjection. After selection, cell lines were continuously kept in medium with G-418.

DNA Reextraction [Modified Hirt Method (18, 19)], Cellular DNA Isolation, Southern Blot Hybridization, and RNA Dot Blot Analysis. For DNA and RNA extraction experiments, rat and mouse cells were grown on small glass slides (2 × 2 mm). After microinjection, cells were further cultivated under standard conditions. Before lysis, slides with the cells were washed in PBS and transferred into Eppendorf tubes filled with 200 μl of lysis buffer (0.6% $NaDodSO_4$/10 mM EDTA, pH 7.5). Tubes were left at room temperature for 10–15 min and 50 μl of 5 M NaCl was added. Cellular DNA was precipitated at 4°C for at least 8 hr. After centrifugation for 15 min in an Eppendorf centrifuge, supernatant was collected and plasmid DNA was precipitated by addition of 3 vol of ethanol and 1/10th vol of 1 M NaCl. The dried DNA pellet was dissolved in TE buffer (10 mM Tris·HCl, pH 7.4/1 mM EDTA). DNA was further purified by RNase treatment and phenol extraction. After digestion with restriction endonucleases, DNA was separated by standard agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized with nick-translated ^{32}P-labeled HSV TK DNA (specific activity, 1–2 × 10^8 cpm per μg of DNA) (20).

For RNA dot blot analysis, microinjected cells were transferred into tubes filled with 50 μl of buffer containing proteinase K (200 μg of proteinase K per ml in 10 mM Tris·HCl, pH 7.0/5 mM EDTA/0.1% $NaDodSO_4$/50 μg of tRNA per ml) and lysed by incubation at 37°C for 15 min. After addition of 1/10th vol of 2.5 M sodium acetate (pH 6.0), the lysate was twice extracted with phenol/chloroform (1:1, vol/vol) and ethanol-precipitated. For denaturation, the pellets were suspended in 20 μl of a solution containing 50% formamide and 6% formaldehyde in TBE buffer (90 mM Tris·HCl/90 mM boric acid/2.5 mM EDTA, pH 8.0) and incubated at 65°C for 3 min. After addition of 180 μl of 6× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl/0.015 M sodium citrate), the solution was filtered on a nitrocellulose sheet, washed under high stringency, and probed with nick-translated HSV TK DNA.

High molecular weight DNA was isolated from the culture cells by a standard procedure (21). Genomic DNA (5–10 μg) was digested with restriction endonucleases, and the product was analyzed by electrophoresis on agarose gels and Southern blotting by standard techniques (22).

RESULTS

The Biological Activity of Methylated HSV TK DNA. The TK plasmids (pHSV-106, pML-BPV-TK4) used in these studies were methylated *in vitro* with the *Hpa* II enzyme. The completeness of methylation was confirmed for each preparation by *Hpa* II and *Msp* I endonuclease digestion and DNA blot analysis as described (13). If not indicated otherwise, 20–40 DNA molecules were microinjected into the nuclei of TK$^-$ rat 2 and mouse LTK$^-$ cells. As summarized in Table 1, the methylated TK DNA (pHSV-106-CH_3) was as biologically active as the mock-methylated TK DNA during the first 48 hr after microinjection as analyzed by [^{3}H]thymidine incorporation and autoradiography. Only with prolonged cultivation of cells does the inhibitory effect of DNA methylation become demonstrable. Cells incubated with [^{3}H]thymidine 100–120 hr after injection did not exhibit TK activity in detectable amounts.

To determine more precisely the time point of transition from methylation insensitivity to sensitivity of the HSV TK gene, the transcription rate of the injected DNA was analyzed. For this purpose, 100 rat 2 cells grown on small glass slides (23) were microinjected with 2–4 DNA molecules each. At the time indicated (Fig. 1), total RNA was extracted from the cells and analyzed by dot blotting using the isolated nick-translated ^{32}P-labeled HSV TK DNA as a hybridization probe. Transcription of the injected DNA was first demonstrable 1–2 hr after the transfer of either methylated or mock-methylated HSV TK DNA. With further cultivation of the injected cells, the rate of HSV TK RNA synthesis increased continuously up to 8 hr. After this time, a sharp decrease of RNA synthesis occurred in cells injected with the methylated DNA. RNA extracts obtained 16 hr after injection did not contain TK RNA over the background, while expression of the mock-methylated DNA continued as expected. We further asked whether enhanced transcription of the TK gene, mediated by the BPV DNA (24), can prolong expression of the methylated DNA over this time. As with the methylated pHSV-106 DNA, inhibition of the pML-BPV-TK4-CH_3 plasmid DNA occurred 8 hr after microinjection.

Biological Activity of HSV TK Chromatin. The experimental results described above have proven that DNA methylation *per se* does not block TK gene expression but requires a further event(s) that occurs later after microinjection. This might be chromatin formation or the integration of the injected HSV TK DNA into the host genome. We have indirect evidence that chromatin formation plays a critical role for this inhibition effect. Our recent results have shown that assembling of the DNA into chromatin also occurs 8 hr after microinjection (25). This 8-hr latency period is presumably due to the low pool of free histone molecules in mammalian cells during interphase (26, 27). To prove more directly that chromatin formation mediates inhibition of the

Table 1. TK activity in microinjected rat 2 cells

Material injected	[^{3}H]Thymidine incorporation at different times after injection		
	0–24 hr	24–48 hr	48–72 hr
pHSV-106	120–140	>200	500*
pHSV-106-CH_3	120–140	160–180	<0.5
pHSV-106 chromatin	120–140	>200	500*
pHSV-106-CH_3 chromatin	0	0	0
pHSV-106 chromatin/ pHSV-106-CH_3 chromatin†	120–140	>200	500*
pHSV-106-CH_3 reextracted from pHSV-106-CH_3 chromatin‡	120–140	160–180	<0.5

After microinjection, cells were labeled with [^{3}H]thymidine (1 μCi/ml) for 24-hr intervals at the time indicated and processed for autoradiography. The number of injected cells was counted as 100%. Data given are average values from five independent experiments with 50 injected cells each and are expressed as % TK$^+$ cells.

*The number of positive cells (48–72 hr after injection) was estimated and not counted.

†pHSV-106-CH_3 chromatin (0.01 mg/ml) was mixed before microinjection with mock-methylated chromatin (0.01 mg/ml) in a 1:1 ratio (vol/vol).

‡pHSV-106-CH_3 DNA was reextracted from the reconstituted chromatin by $NaDodSO_4$/phenol treatment.

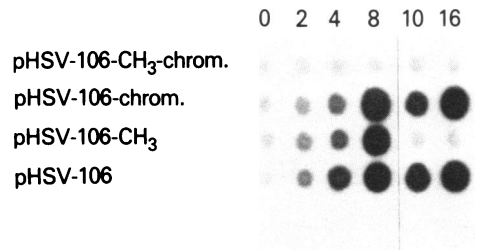

FIG. 1. HSV TK RNA dot blot. Total cellular RNA was isolated from rat 2 cells at various times, shown in hours, after microinjection of either methylated or mock-methylated HSV TK DNA and chromatin.

transcription, we analyzed the biological activity of methylated and mock-methylated HSV TK chromatin. As a model system, we first attempted to methylate purified polyoma virus minichromosomes with *Hpa* II methylase. We found that *in vitro* methylation of chromatin is a very inefficient process. Even at exhaustive incubation conditions of 3–4 days and a 10- to 20-fold excess of the enzyme concentration required for full DNA methylation, only a small number of chromatin molecules were methylated (data not shown).

As an alternative approach, we decided to reconstitute chromatin *in vitro* with isolated histone molecules. As shown by workers in different laboratories, chromatin formation can be efficiently obtained *in vitro* by using purified histone octamers for the reconstitution (16, 17, 28). We isolated the histone octamers from chicken embryos as described in *Materials and Methods*. The purified octamers were mixed with either methylated or mock-methylated HSV TK DNA in a 1:1 ratio (wt/wt) and chromatin assembly was obtained during dialysis. Fig. 2 shows an electron micrograph of

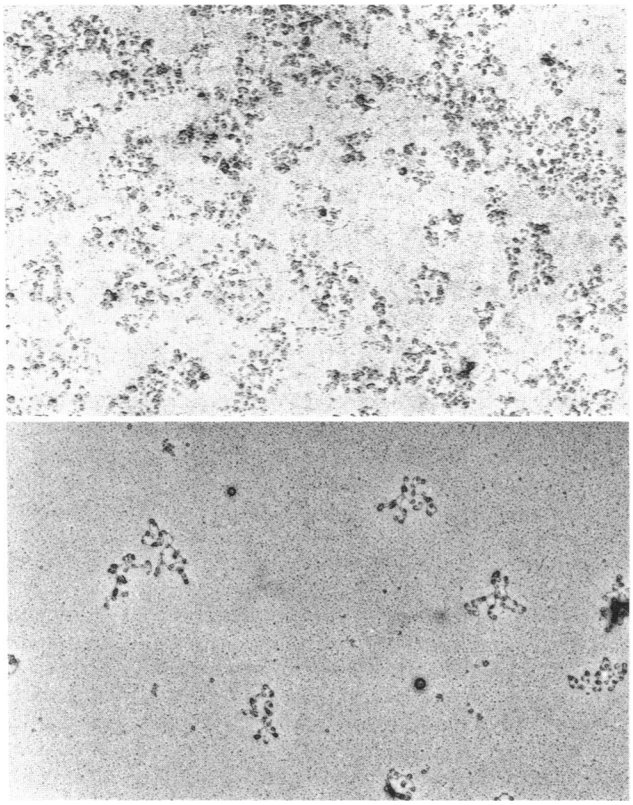

FIG. 2. Electron micrographs of the *in vitro* reconstituted pHSV-106-CH_3 chromatin. (*Lower*) Chromatin molecules from the same chromatin preparation diluted a further 1:20 before processing for electron microscopy.

reconstituted HSV TK chromatin molecules. A great majority of the molecules are in a chromatin-like structure, and free DNA molecules are almost not detectable. The HSV TK DNA is tightly packaged with histone cores, forming beads of the nucleosomal size. On the average, 20–26 beads are found per circular molecule. The *in vitro* reconstituted chromatin also behaves as native chromatin when treated with micrococcal endonuclease. Limited digestion generated a typical nucleosomal DNA fragment pattern for both methylated and mock-methylated HSV TK chromatin (data not shown).

To test the biological activity of the reconstituted chromatin, 20–40 molecules were microinjected into the nuclei of rat 2 cells. At different times after injection (Table 1), cells were labeled with [^{3}H]thymidine for 24-hr intervals and processed for autoradiography. As summarized in Table 1, the reconstituted chromatin was as active as the pHSV-106 DNA itself. Also, the rate of HSV TK RNA synthesis was similar in cells injected with the chromatin as with the DNA (Fig. 1). To exclude the possibility that this activity was caused by contamination of the chromatin preparation with a small number of free or not fully reconstituted DNA molecules, the experiments described above were repeated with a lower chromatin concentration. After microinjection of only one or two chromatin molecules per cell, a high HSV TK activity was also observed. We compared further the transformation efficiency of the reconstituted chromatin with that of the HSV TK plasmid DNA. Following intranuclear injection of 20–40 HSV TK DNA molecules per cell, 20–30% of the recipients were converted into permanent TK^+ cell lines. This high transformation efficiency was also obtained after microinjection of 20–40 reconstituted chromatin molecules (pHSV-106 and pML-BPV-TK4) (Table 2). In all analyzed TK^+ rat 2 cell lines, the HSV TK DNA was found to be covalently integrated into the host genome.

Finally, we analyzed the biological activity of the methylated chromatin. In contrast to the results obtained with the mock-methylated chromatin, the methylated pHSV-106 and pML-BPV-TK4 chromatin were always inactive after microinjection. Also, after the transfer of 200–400 molecules per cell, TK activity was not demonstrable by either autoradiography or by RNA dot analysis (Fig. 1). These results showed that the nucleosomal structure of the methylated DNA was crucial for the inhibition of TK gene expression, although a further involvement of nonhistone proteins cannot yet be excluded.

To exclude the fact that the inactivity of the methylated chromatin was caused by DNA degradation, the HSV TK DNA was reextracted from the recipient cells by the Hirt method (19) 2, 4, and 6 hr after chromatin injection. Southern blot analysis of the DNA extracts revealed that the cells still contained significant amounts of the HSV TK DNA 6 hr after

Table 2. Transformation efficiency of methylated and mock-methylated chromatin after microinjection into rat 2 or LTK^- cells

Material injected	% stable transformed clones selected	
	HAT medium	G-418
pHSV-106 chromatin	20–30	15–20
pHSV-106-CH_3 chromatin	0	15–20
pML-BPV-TK4 chromatin	20–30	NT
pML-BPV-TK4-CH_3 chromatin	0	12–20

Single cells grown on small glass slides subdivided into numbered squares (18) were microinjected into the nuclei with 20–40 molecules each. One day after injection, slides with the cells were transferred into hypoxanthine/aminopterin/thymidine (HAT) medium or into medium with G-418. Three weeks after injection, the number of positive clones were counted. When grown in mass culture, cells were kept under selective pressure. NT, not tested.

chromatin injection (Fig. 3). These results indicate further that the reconstituted chromatin remains stable after microinjection, since dissociation of one single chromatin molecule into methylated DNA and histones would be sufficient to make the recipient cell TK positive and thus detectable by autoradiography. The HSV TK DNA was also demonstrable late after microinjection of the methylated chromatin into rat 2 cells. Cell lines obtained by the drug G-418 selection following coinjection of the chromatin (pHSV-106-CH_3 or pML-BPV-TK4-CH_3) with the pCG-BPV9 DNA (this plasmid encodes resistance for the antibiotic G-418 and contains the BPV DNA) (29) contained the HSV TK DNA covalently integrated in the host genome and still methylated. This was tested by Southern blot analysis of *Hpa* II and *Msp* I restricted cellular DNA, which was isolated from the cells at passage number 12 after microinjection and hybridization to ^{32}P-labeled pHSV-106 DNA. Addition of 5-azacytidine (5 μM for 48 hr) to the culture medium of the transformed cells induced reactivation of the HSV TK gene and subsequent growth of the cells in HAT medium. This activation process was linked to demethylation of the HSV TK DNA (data not shown).

To clarify the question of whether integration of the methylated HSV TK gene into the host genome is also required for inhibition of HSV TK DNA transcription, the transformation experiments described above were repeated with the methylated pML-BPV-TK4 chromatin using the mouse LTK$^-$ cells instead of rat 2 cells as recipients. As shown recently, plasmid molecules containing the BPV DNA, as the pML-BPV-TK4 does, remain in an episomal state in mouse cells after DNA transfection (15, 29). Following coinjection of 100 LTK$^-$ cells with the methylated chromatin and the pCG-BPV9 DNA, 12 independent cell lines were obtained by G-418 selection. At passages 8–10, the DNA was extracted by the Hirt method from cells of three arbitrarily chosen lines and analyzed by Southern blotting. As estimated from the intensity of the pML-BPV-TK4-specific DNA bands on the blot (Fig. 4), the transformed cells contained 20–30 episomal pML-BPV-TK4 molecules per cell still methylated. None of them expressed the HSV TK gene, as tested by autoradiography of the [^{3}H]thymidine-labeled cells and RNA dot blot analysis. However, between passages 15 and 18, cells of two independent lines started to grow spontaneously in HAT medium. Activation of the HSV TK gene in these cells was connected with demethylation of the episomal pML-BPV-TK4 chromatin (Fig. 4).

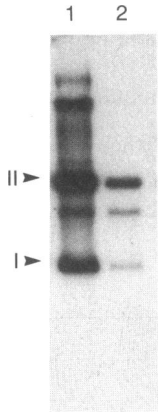

FIG. 3. Southern blot of pHSV-106-CH_3 DNA reextracted from 300 rat 2 cells by the Hirt method 6 hr after pHSV-106-CH_3 chromatin injection (lane 1), and as marker, the pHSV-106-CH_3 DNA reextracted from the reconstituted chromatin by NaDodSO_4/phenol treatment (lane 2). For DNA reextraction experiments, 2000–4000 molecules were injected per cell (10). The positions of the superhelical DNA I and the relaxed circular form II are indicated.

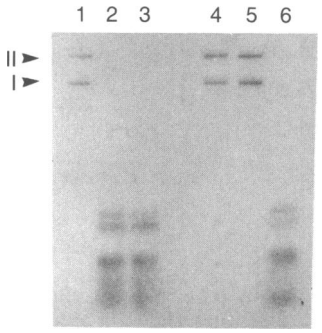

FIG. 4. Southern blot of extrachromosomal pML-BPV-TK4 DNA extracted from transformed mouse cells at passage 8 (lanes 4–6) and passage 17 (lanes 1–3). Isolated DNA (lanes 1 and 4) was digested with either *Hpa* II (lanes 2 and 5) or with *Msp* I (lanes 3 and 6) endonucleases.

DISCUSSION

Our microinjection experiments have shown that DNA methylation *per se* does not prevent the expression of the HSV TK gene. The methylated HSV TK DNA was as active as the mock-methylated DNA for several hours after microinjection into the nuclei of TK$^-$ mouse and rat cells. The switch from methylation insensitivity to sensitivity correlated in time with chromatin formation of the injected DNA (25). Thus, it is obvious that the methylcytosines cannot effect the interplay of the DNA with RNA polymerase and transcription factors until chromatin formation occurs. Because mammalian cells have a low free histone pool during interphase, efficient chromatin formation is only possible after the recipient cells have entered into S phase (30).

That chromatin formation indeed mediates the inhibitory effect of DNA methylation on gene expression was directly demonstrated by microinjection of *in vitro* reconstituted HSV TK chromatin. As tested by autoradiography of the [^{3}H]thymidine-labeled cells (Table 1) and by RNA dot analysis (Fig. 1), the methylated chromatin was found always to be inactive. However, this inactivity is reversible; exposure of the cells to 5-azacytidine or spontaneous demethylation of the chromatin caused HSV TK gene expression and growth of the cells in HAT medium. *In vitro* reconstitution of methylated and mock-methylated HSV TK DNA with purified chicken histone octamers was chosen since *in vitro* methylation of intact chromatin is a very inefficient process. As shown in this investigation and elsewhere, *in vitro* reconstituted chromatin behaved under all analyzed aspects as native isolated chromatin (17, 28).

Our results document further that chromatin formation and not integration into the host genome plays the key role in this regulatory process. Mouse cells that contain the methylated HSV TK chromatin in an episomal state mediated by the BPV DNA did not express the HSV TK gene. This shows that the formation of a higher chromatin structure, as the 30-nm chromatin fiber (6), is not required to block transcription of the methylated chromatin.

This leads to the question of which feature of the chromatin mediates this inhibitory effect. Different groups have tried to characterize active chromatin in further detail (31–33). So far it has not been possible to obtain a clear understanding of the biochemical and morphological properties that specify active versus inactive chromatin. However, there are several features that are frequently associated with active chromatin. One feature, the DNase I hypersensitive sites, are located predominantly at the 5′ region of genes (33, 34). The nature of these hypersensitive sites is still uncertain but may reflect a change in the protein composition and an alteration of the DNA and chromatin structure in these particular regions.

Recent experiments have suggested that modulation of torsional strain and the association of DNA with topoisomerase I and II could play a role in the creation of the nuclease sensitivity (35–38). Furthermore, a direct correlation between undermethylation and DNase I sensitivity was demonstrated (for review, see ref. 31). In this regard, it is of interest that Keshet *et al.* (39) found that the methylated inactive HSV TK chromatin was free of hypersensitive sites late after DNA transfection into mouse cells. If DNA methylation prevents the generation of DNase hypersensitive sites and, hence, gene expression, then there are exceptions to this rule. We have shown that SV40 DNA fully methylated by rat liver methylase was also active late after injection into rat and monkey cells (10). This could mean that either the methylated SV40 chromatin acquires DNase I hypersensitivity in spite of a high number of methylated cytosines or the DNase hypersensitivity is not essential for SV40 gene expression.

A further characteristic of SV40 chromatin is the nucleosome-free region of ≈400 base pairs around the early promoter region (40, 41). The relevance of this gap for gene expression is still a matter of speculation (36). It may be that this nucleosome-free region mediates insensitivity of the methylated SV40 chromatin, allowing transcription to occur as it would with the injected free DNA.

After *in vitro* reconstitution, we did not observe a significant difference between methylated and mock-methylated chromatin, as tested by electron microscopy and micrococcal endonuclease treatment. However, it will be important to test whether generation of DNase hypersensitive sites and formation of nucleosome-free regions will occur following microinjection of the methylated and mock-methylated HSV TK and SV40 chromatin into culture cells.

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